between the present application and the '774 patent. Claims 34, 35, 37-39, 41-43 and 45 provided that the amplification products of the target nucleic acid sequence and the control sequence be distinguishable by size. As described in the specification and claims of the parent application no. 07/402,450, the amplification products of the target sequence and the control sequence may also be distinguished using an internal oligonucleotide probe. Accordingly, claims 46-48, which are presented herein, specify that the amplificaiton products of the target and control sequences are distinguishable by size or by use of an internal oligonucleotide probe. These claims otherwise are identical to claims 34, 35 and 37, respectively. Support for this limitation appears in the specification, for example at page 7, lines 20-22 and in original claims 18, 26 and 27.

Newly added claim 49 is directed to a method for the quantitative determination of a target nucleic acid sequence. Support for this claim appears in the specification, for example at page 6, line 15 through page 8, line 4.

In the preliminary amendment filed on December 18, 1996, claim 15 of the '774 patent was proposed as the interference count. Each of newly added claims 46-49 should be designated as corresponding to the count. Compliance with 35 U.S.C. § 135(b) is satisfied with respect to newly added claims 46-49, inasmuch as they are being submitted within one year of the December 19, 1995 issue date of the '774 patent.

Applicants are entitled to the benefit of the January 27, 1988 filing date of grandparent application serial no. 07/148,959 (the "'959 application") (attached as App. B to the December 18, 1996 preliminary amendment) with respect to claims 46-59. Support for those claims is

found, for example, at page 3, line 27 through page 4, line 2 and page 5, lines 1-11of the '959 application.

In view of the foregoing, Applicants respectfully request entry of the foregoing amendments and declaration of interference with the '774 paeent.

Respectfully submitted,

By _____

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APPENDIX B

PATENT APPLICATION OF

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FOR

SIMULTANEOUS AMPLIFICATION OF CERTAIN RNA SEQUENCES

SIMULTANEOUS AMPLIFICATION OF CERTAIN RNA SEQUENCES

This invention relates to the detection and quantification of viral RNA from peripheral blood samples or H-9 cells by simultaneous amplification with at least one other RNA sequence.

BACKGROUND OF THE INVENTION

Co-pending Murakawa et al application Serial No. 941,379 filed December 15, 1986 describes a procedure for the amplification of small quantities of viral RNA. The Murakawa procedure comprises adding to RNA two converging oligodeoxyribonucleotide primers at least one of which is complementary to a sequence included in the RNA; thereafter extending at least one of the primers with one or both of reverse transcriptase and DNA polymerase I; and determining whether the sequence is present in the amplified segment by use of a synthetic oligodeoxyribonucleotide probe complementary to a region within the segment. The specification and claims of application Serial No. 941,379 in their entirety are by express reference incorporated herein and made a part hereof.

This invention adapts the Murakawa two primer amplification procedure to the identification and quantification of viral RNA present in peripheral blood samples and H-9 cells. More particularly such RNA is amplified simultaneously with at least one other RNA sequence, the amplification product of which provides a positive control useful to reveal false negative data indicating the absence of viral RNA. The invention includes a kit comprising reagents appropriate for use in its practice.

DETAILED DESCRIPTION OF THE INVENTION

To provide a positive control, at least one synthetic RNA sequence is amplified simultaneously with RNA from a virus affected T-4 lymphocyte or H-9 cell present in a peripheral blood sample.

A first primer pair includes an oligonucleotide sequence effective to amplify viral RNA. For AIDS virus (HIV-1), preferred primers are synthetic oligonucleotides including the following sequences which occupy positions within the HIV-1 3' ORF region:

HIVA: 5'ATGCCGATTGTGCTTGGCTA 3'
HIVB: 5'TGAATTAGCCCTTCCAGTCC 3'

A preferred HIV-1 hybridization probe includes the sequence:

HIVC (PROBE): 5'AAGTGGCTAAGATCTACAGCTGCCT 3'

The process of this invention can also be applied to human cytomegalovirus (HCMV). A target for amplification is a region of the HCMV major IE gene (IE1) region between nucleotides 1154 and 1331. Oligodeoxyriboribonucleotides complementary to sequences in this region are used with RNA from HCMV infected cells, and with RNA from patient samples. Suitable oligonucleotide primers and probes have the following sequences:

HCMVA	1154	5' CGAGACACCCGTGACCAAGG 3'	1173
HCMVB	1311	3' CTCTTTCTACAGGACCGTCT 5'	1330
HCMV (Probe)	1182	3' AAGGACGTCTGATACAACTCCTT 5'	1204

An additional amplification system is needed for detection of RNA from transcription of late HCMV genes, which is an important marker for active infection. Sequences 866-1025 from the coding sequence of p64 are amplified. Suitable oligonucleotide primers and probes have the following sequences:

HCMVD	866	5' AAAGAGCCCGACGTCTACTACACGT 3'	890
HCMVE	1001	3' CTGGTCATGCAGTTCCACATGGACC 5'	1025
HCMV Probe II	941	3' CGCGTGCTCGACCAAACGAGGTACCTCTTG 5'	970

The cell population primarily affected by a virus, e.g., the AIDS virus, is the T-4 lympohcyte population which, like other T-cells express the T-cell receptor.

Accordingly, a second primer pair includes an oligonucleotide to amplify a sequence which is unique to the T-cell receptor. Although other such unique sequences may be selected, preferably the second primer is effective to amplify the constant region of the T-cell receptor beta chain. For HIV-1, T-cell receptor primers A and B are preferred.

T-Cell Receptor A: 5'GTCCACTCGTCATTCTCCGA 3'
T-Cell Receptor B: 5'TCAAGACTCCAGATACTGCCT 3'

A preferred T-cell receptor hybridization probe includes the following sequence:

T-Cell Receptor C (PROBE): 5'CAGAAGGTGGCCGAGACCCTCAGGC 3'

A third primer pair is effective to amplify an RNA sequence present, preferably ubiquitously, in all of the cells of a peripheral blood sample or of an H-9 cell sample, even when the T-cell count is low. Preferably the third primer pair amplifies beta actin sequence. Synthetic oligonucleotides comprising the following sequences are preferred:

Beta Actin A: 5'CTCATTGCCAATGGTGATGACCTG 3'
Beta Actin B: 5'GCTATCCCTGTACGCCTCTGGC 3'

A preferred beta actin hybridization probe includes the following sequence:

Beta Actin C (PROBE): 5'CGGTGAGGATCTTCATGAGGTAGTC 3'

A fourth primer to provide an additional aid to quantitation of virus levels in patient samples is provided by a reference RNA which can be amplified and detected by the same oligonucleotides used for authentic virus RNA samples.

For HIV-1 such a reference RNA may be a "maxigene" formed by a multi-base pair insert into a unique site, for example the unique KpnI site of the 3' ORF region. A preferred reference RNA includes a 22 base pair insert into the KpnI site of the HIV-1 3' ORF region of the pGEM92 clone described by Murakawa. An insert of sequence: 5'CACACAAGGCTACTTCGGTAC, CATGGTGTGTTCCGATGAAGC5' is appropriate. The underlined

sequences present in the AIDS virus bracket the 22 base pair insert.

The pGEM92 clone is produced using as a starting material the plasmid pSP64-BH10-R3 (Biotech Research Laboratories, Inc.) which contains the entire HIV virus excluding the LTRS. A 1.1 Kb Bam H1 restriction fragment including HIV sequences 8052 to 9149 was subcloned in both orientations into the Bam H1 site of the transcription vector pGEM2 (Promega Biotech). The resulting plus strand plasmid is designated pGEM92. The 22 base pair sequence is inserted in known manner.

The transcription product of this clone is 22 bases longer than the authentic HIV-sequence but still hybridizes with the 25 mer probe HIVC. It is therefore distinguishable by size from the authentic viral product.

For purposes of identification and quantification, the amplification products are electrophoresed in a gel, e.g., agarose or 6% polyacrylamide, 8 M. urea gel. Labelled probes complementary to each of the amplified sequences are used sequentially. Hybridization of the probes with amplification products other than of authentic HIV provides positive controls thus minimizing the possibility of false negative data regarding the authenticity of the original sample. More particularly if the HIV probe yields negative data, but one or both the T-cell receptor and beta actin probes yield positive data, the conclusion may be feasibly drawn that the original sample was viable notwithstanding the negative HIV probe result.

The "maxigene" provides an internal control and an additional aid to quantitation. Because the quantity of "maxigene" RNA originally included in the amplification reaction is known, the amount of signal obtained from this amplification product can be related to the signal obtained from the patient sample. Hence, the relative quantitation of the original amount of authentic HIV-1 in the patient sample is also provided by this construct.

To provide appropriate signals either the primers or the probes are labelled, e.g., with an isotope such as P^{32} or a fluorescent. Preferably, the probes are labelled.

Amplification using an oligonucleotide primer containing the T-7 RNA polymerase (Biorad Laboratories) increases the sensitivity of detection. The following sequence HIV T7 is illustrative:

HIV T7 - 5'TTAATACGACTCACTATAGGGATGCTGATTGTGCCTGGCTA 3'

Figure 1 exemplifies simultaneous priming with HIVA and B and T-cell receptor A and B synthetic oligonucleotides. Figure 1A shows the results with HIVC probing. Figure 1B shows the results with T-cell receptor probing.

Figure 2 shows that HIV oligonucleotide primers and probes have homology with the human cytomegalovirus (HCMV) suggesting that HIV-1 has homology with an actively transcribed region of HCMV.

EXEMPLIFICATION OF THE INVENTION

Example I

Amplification is performed using 1X amplification buffer (10 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 66 mM NaCl; 1 mM dithiothreitol), 1.5 mM of each dNIP). To this buffer, about 1 mM total peripheral blood lymphocyte RNA from an AIDS infected patient and about 1.0 mM of each of the priming nucleotides HIVA, HIVB T-cell receptor A and T-cell receptor B

are added providing a final reaction volume of approximately 100 µl. The sample is heated at 95°C for 2 minutes, centrifuged for 5 seconds, cooled to 37°C for about 2 minutes at which time 1.0 µl of AMV reverse transcriptase (Life Sciences or BioRad Laboratories) diluted in the amplification buffer were added and incubation was continued for 2 minutes at 37°C. A second ampification cycle was preformed in like manner. Thereafter the final 28 rounds of amplification were accomplished using a buffer consisting of 2.5 units of Thermus aquatus DNA polymerase (Perkin-Elmer Cetus or New England Biolabs): 50 mM KCl, 10 mM Tris, pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 µM each dNTP, and 50 pmoles of each primer in a final volume of 50 microliters overlain with 10 microliters of paraffin oil. The polymerizations are carried out from 1 to 2 minutes at 65°C, with 1 minute of denaturation at 95°C, and 1 minute of annealing at 37°C.

After completion of the last cycle of amplification, the products are placed on ice and a 10 µl portion was electrophoresed in a 1.8% agarose gel. The DNA was transferred to Zeta probe (Biorad) using an alkaline blotting procedure and prehybridized and hybridized as follows: The prehybridization reaction was performed at 65°C for 1 to 3 hours in 20 ml of 6X SSPE (1.0 M NaCl, 0.06 M $NaPO_4$, 0.006 M EDTA); 1.0% SDS; 0.5% rehydrated, powder skim milk (Alba); and 10 µg per ml of sonicated, denatured salmon sperm DNA. The hybridization reaction was in 20 ml of the same buffer, except the salmon sperm DNA was omitted and replaced with 20 pmol of 5'-^{32}P-labelled oligodeoxyribonucleotide HIVC (ca. 3 x 10^8 cpm). Hybridization was for 1 hour to overnight at 65°C. The hybridized filter was washed with three 250 ml volumes of 6X SSC (0.95 M NaCl, 0.095 M Na Citrate), 0.1% SDS at 65°C for 5

minutes each, and autoradiographed at -70°C for 1 hour on Kodak XAR-5 film with an intensifying screen.

Each of the HIVC and T-cell receptor C probes is used separately and sequentially. After the results with the HIVC probe are obtained, that probe is stripped from the filter by treatment with 100C 0.1 X SSC, 0.1% SDS, two times for 15 minutes each. The filter is then rehybridized to the T-cell receptor C probe.

Bands from each of the authentic HIV and T-cell receptor samples are detected after Southern Blot hybridization.

EXAMPLE II

Example I is repeated with the exception that the primer pair beta actin A and beta actin B is included in the amplification reaction mixture.

The amplification products are analyzed separately and sequentially by probes which hybridize with authentic viral RNA, the amplified T-cell receptor RNA sequence and the amplified beta action A sequence. Bands from each such sequence are detected after Southern blot hybridization.

EXAMPLE III

Example I is repeated with the exception that the maxigene primer is included in the reaction mixture.

Kits contemplated by the invention include self-contained appropriate quantities of primers and probes for use in the practice of the invention.

WE CLAIM:

1. A process for identifying a viral RNA nucleotide sequence present in a sample of peripheral blood or H-9 cells which comprises amplifying such RNA simultaneously with at least one other RNA nucleotide sequence present in a virus infected cell in said sample, and thereafter separately and sequentially analyzing the amplification reaction products with probes homologous with authentic RNA and with such other RNA sequence to identify one or both of said RNA nucleotide sequences.

2. The process of claim 1 in which the viral RNA is HIV-1 RNA.

3. The process of claim 1 in which the viral RNA is HCMV RNA.

4. The process of claim 1 in which a third RNA nucleotide sequence ubiquitously present in substantially all of the cells of said sample is included in the amplification reaction and in which the analysis of the amplification product includes a third probe to identify said third nucleotide sequence.

5. The process of claim 4 in which the viral RNA is HIV-1 RNA.

6. The process of claim 4 in which the viral RNA is HCMV RNA.

ABSTRACT

A process for identifying a viral RNA nucleotide sequence present in a sample of peripheral blood or H-9 cells which comprises amplifying such RNA simultaneously with at least one other RNA nucleotide sequence present in a virus infected cell in said sample, and thereafter separately and sequentially analyzing the amplification reaction products with probes homologous with authentic RNA and with such other RNA sequence to identify one or both of said RNA nucleotide sequences.

FIG. 1A FIG. 1B

FIG. 1A - AIDS PROBE
FIG. 1B - T-CELL RECEPTOR PROBE

H9 Cells Infected with AIDS.
P = AIDS Patient Sample.

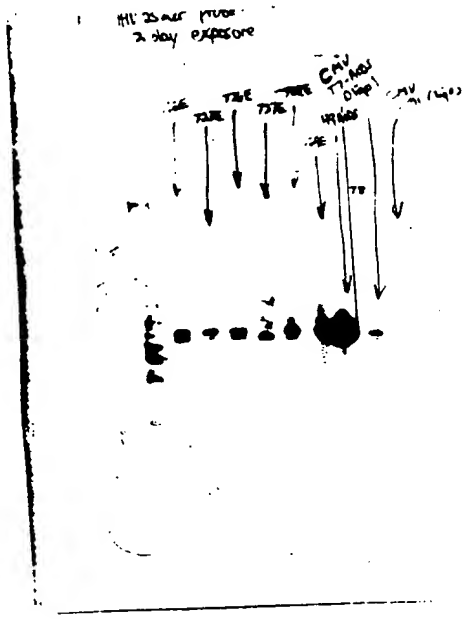

FIGURE 2